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Direct Measurement of NAD(P)H:Quinone Reductase from Cells Cultured in Microtiter Wells: A Screening Assay for Anticarcinogenic Enzyme Inducers

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We describe a rapid and direct assay of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) activity in cultured cells suitable for identifying and purifying inducers of this detoxication enzyme. Hepa 1c1c7 murine hepatoma cells are plated in 96-well microtiter plates, grown for 24 h, and exposed to inducing agents for another 24 h. The cells are then lysed and quinone reductase activity is assayed by the addition of a reaction mixture containing an NADPH-generating system, menadione (2-methyl-1,4-naphthoquinone), and MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide]. Quinone reductase catalyzes the reduction of menadione to menadiol by NADPH, and MTT is reduced nonenzymatically by menadiol resulting in the formation of a blue color which can be quantitated on a microtiter plate absorbance reader. The reaction is more than 90% dicoumarol inhibitable and menadione dependent. The results are comparable to those obtained by harvesting cells from larger plates, preparing cytosols, and carrying out spectrophotometric measurements.

KEY WORDS: quinone reductase; phase II enzymes; enzyme induction; microtiter plates; anticarcinogens.

We have developed a rapid, efficient, and inexpensive assay for measuring NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) from cells cultured in microtiter wells. Quinone reductase[2] is a widely distributed, primarily cytosolic, dicoumarol-inhibitable flavoprotein that catalyzes the reduction of a wide variety of quinones and quinoneimines (1,2). Quinone reductase protects cells against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones which are then susceptible to glucuronidation (3–10). In addition, quinone reductase is induced coordinately with other electrophile-processing Phase II enzymes (glutathione *S*-transferases and UDP-glucuronosyltransferases) by a variety of compounds that protect rodents from the toxic, mutagenic, and neoplastic effects of carcinogens (2,11–13). There is a large body of evidence which suggests that the induction of Phase II enzymes is the predominant mechanism by which these heterogeneous compounds are chemoprotective (11–15), and it is clear that the monitoring of Phase II enzyme induction is a convenient method for screening for anticarcinogenic activity (11–13,15–23).

Although many anticarcinogenic enzyme inducers have been discovered, other unrecognized compounds may exist that are potent, effective, and nontoxic (e.g., the active constituents from poorly characterized plant

[1] To whom reprint requests should be addressed.

[2] Abbreviations used: quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2); DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; Sudan I, 1-phenylazo-2-naphthol; Sudan II, 1-(2,4-dimethylphenylazo)-2-naphthol; Sudan III, 1-(4-phenylazophenylazo)-2-naphthol.

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extracts) (11,17,20–23). Unfortunately, screening compounds for their ability to induce Phase II enzymes in animals is difficult, time-consuming, and expensive (17,20). Our laboratory has recently developed a more rapid screening system by demonstrating that quinone reductase is induced in the Hepa 1c1c7 murine hepatoma cell line by many of the compounds that induce Phase II enzymes *in vivo* (24–26). Nevertheless, conventional assay techniques (e.g., harvesting, homogenizing, centrifuging, and assaying for enzymatic activity and protein content) are time-consuming and therefore limit the usefulness of this procedure. For this reason, we have developed a direct assay of quinone reductase from cells grown and induced in 96-well microtiter plates by measuring the NADPH-dependent menadiol-mediated reduction of MTT. This assay procedure is rapid, accurate, inexpensive, capable of screening many compounds and/or a series of concentrations of compounds in a single experiment, and amenable to computerized data processing. This method should facilitate the identification of new and potentially important chemoprotective compounds of medicinal interest.

EXPERIMENTAL PROCEDURES

Materials

MTT, NADP, FAD, menadione, bovine serum albumin, Tris base, glucose 6-phosphate, bakers' yeast glucose-6-phosphate dehydrogenase, Tween-20, penicillin G, streptomycin, and crystal violet were obtained from Sigma Chemical Co. (St. Louis, MO); NADH was from Pharmacia P-L Biochemicals (Piscataway, NJ); microtiter wells were from Falcon (Becton–Dickinson Labware, Oxnard, CA); α-minimal essential medium and fetal calf serum were from GIBCO (Grand Island, NY); and DMSO and acetonitrile were from Burdick and Jackson (Muskegon, MI). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from IIT Research Institute (Chicago, IL). Other inducing agents were obtained and prepared as described previously (24–26). Hepa 1c1c7 cells were a gift of J. P. Whitlock, Jr., Stanford University. Multiple pipettors (50- and 200-μl Octapipets) were purchased from Costar (Cambridge, MA). The absorbances of microtiter wells were measured with an automated optical scanner equipped with a 610-nm filter (Biotek, Winooski, VT).

Methods

Growth of cells. Hepa 1c1c7 cells were plated at a density of 10 000 cells/well in 200 μl of α-minimal essential medium (without ribonucleosides or deoxyribonucleosides) supplemented with 10% fetal calf serum. The cells were grown for 24 h in a humidified incubator in 5% CO_2 at 37°C. The medium was decanted and each well was refed with 200 μl of α-minimal essential medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 0.1% DMSO. Compounds to be tested as inducers were dissolved in DMSO and were diluted into the media so that the final concentration of DMSO was 0.1% by volume. Control cells were always grown in the second column of wells and were fed media containing 0.1% DMSO. The cells were then incubated for an additional 24 h.

Assay of quinone reductase. The following stock solution was prepared for each set of assays: 7.5 ml of 0.5 M Tris–Cl (pH 7.4), 100 mg of bovine serum albumin, 1 ml of 1.5% Tween-20, 0.1 ml of 7.5 mM FAD, 1 ml of 150 mM glucose 6-phosphate, 90 μl of 50 mM NADP, 300 U of yeast glucose-6-phosphate dehydrogenase, 45 mg of MTT, and distilled water to a final volume of 150 ml. Menadione (1 μl of 50 mM menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the mixture was dispensed into the microtiter plates.

After the plates were exposed to test compounds for 24 h, the media were decanted, and the cells were lysed by incubation at 37°C for 10 min with 50 μl in each well of a solution containing 0.8% digitonin and 2

mM EDTA, pH 7.8. The plates were then agitated on an orbital shaker (100 rpm) for an additional 10 min at 25°C, after which 200 μl of the complete reaction mixture was added to each well with the aid of a multiple pipetting device (Octapipet). A blue color developed and the reaction was arrested after 5 min by the addition of 50 μl of a solution containing 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4. The plates were then scanned at 610 nm. The first column of wells in the plates always contained the reaction mixture only and served as the nonenzymatic blank. The average absorbance value of this column of wells was subtracted automatically from all other absorbance readings.

In order to determine the proportion of MTT reduction attributable to quinone reductase activity (Table 1), three sets of microtiter plates were grown and induced under identical conditions. The cells on one set of plates were assayed as described above. A second set of cell lysates was assayed in the presence of 50 μl per well of 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4). The third set of cells was lysed in the standard fashion but assayed with a reaction mixture containing no menadione. The absorbances were scanned 5 min after the addition of the reaction mixture.

Crystal violet staining. Since some quinone reductase inducers or crude fractions that are being screened for inducer activity depress the rate of cell growth, it is desirable to relate the observed quinone reductase activity to the number of cells or the amount of protein in each microtiter well. This normalization can be conveniently accomplished by staining a set of microtiter plates treated identically to those used for the MTT assay with crystal violet. We have used a slight modification of the method of Drysdale *et al.* (27) for this purpose. The media were decanted, the plates were submerged in a vat of 0.2% crystal violet in 2% ethanol for 10 min and rinsed for 2 min with tap water, and the bound dye was solubilized by incubation at 37°C for 1 h with 200 μl of 0.5% sodium dodecyl sulfate in 50% ethanol. The plates were then scanned at 610 nm.

In order to demonstrate the validity of the crystal violet assay as a convenient measure of protein content and cell number, five twofold serial dilutions of Hepa 1c1c7 cells were plated in each of six identical 24-well 2-cm² plates (four wells per dilution of cells). The cells were grown for 24 h, refed with

TABLE 1

RATES OF MTT REDUCTION OF CONTROL, β-NAPHTHOFLAVONE OR 1,2-DITHIOL-3-THIONE-TREATED HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS

Treatment of cells	Number of wells assayed	Change in absorbance ($\times 10^3$) in 5 min at 610 nm: Standard assay	Standard assay with prior dicoumarol addition	Standard assay minus menadione
Control	16	212 ± 12[a]	16.1 ± 4.4	22.0 ± 2.8
β-Naphthoflavone (2 μM)	8	862 ± 20	17.3 ± 4.0	17.3 ± 4.0
1,2-Dithiol-3-thione (10 μM)	8	453 ± 20	20.3 ± 3.4	20.3 ± 3.4

Note. Hepa 1c1c7 cells were grown and induced in three parallel sets of microtiter wells as described under Materials and Methods. One set of plates was lysed and assayed in the standard fashion, another set was assayed in the presence of 50 μl of 0.3 mM dicoumarol per well, and the third set was assayed with reaction mixture containing no menadione.

[a] Mean values ± standard deviations.

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COMPARISON OF QUINON
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Compounds

Polycyclic aromatics
2,3,7,8-Tetrachlorodibenz
β-Naphthoflavone
Benzo[a]pyrene
3-Methylcholanthrene

Azo dyes
1,1'-Azonaphthalene
1-(2-Pyridylazo)-2-naphth
1-(2-Thiazolylazo)-2-naph
Sudan I
Sudan II
Sudan III

Diphenols
Catechol
Resorcinol
Hydroquinone
tert-Butylcatechol
tert-Butylresorcinol
tert-Butylhydroquinone

Isothiocyanate
Benzylisothiocyanate

Dithiolthiones
1,2-Dithiol-3-thione
4-Phenyl-1,2-dithiol-3-th
5-(2-Pyrazinyl)-4-methyl-
dithiol-3-thione

[a] Direct assay described
[b] Hepa 1c1c7 cells grow
[c] Mean value ± standard
[d] Unpublished results (N
[e] From Prochaska *et al.*

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with rol	Standard assay minus menadione
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	17.3 ± 4.0
	20.3 ± 3.4

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TABLE 2

COMPARISON OF QUINONE REDUCTASE INDUCTIONS OBTAINED BY DIRECT ASSAY OF HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS AND BY CONVENTIONAL ASSAY OF HEPA 1c1c7 CELLS GROWN ON 75-cm² PLATES

Compounds	Concentration (μM)	Ratio quinone reductase specific activity (treated/control): Direct assay in microtiter wells[a]	Ratio quinone reductase specific activity (treated/control): Conventional assay with 75-cm² plates[b]
Polycyclic aromatics			
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.01	3.10 ± 0.51[c]	2.95 ± 0.40[c,d]
β-Naphthoflavone	2	4.66 ± 0.25	3.56 ± 0.34[d]
Benzo[*a*]pyrene	2	3.80 ± 0.34	3.58 ± 0.08[d]
3-Methylcholanthrene	2	3.43 ± 0.40	3.29 ± 0.24[d]
Azo dyes			
1,1'-Azonaphthalene	2	5.08 ± 0.36	4.47 ± 0.52[d]
1-(2-Pyridylazo)-2-naphthol	2	4.80 ± 1.07	3.61 ± 0.26[d]
1-(2-Thiazolylazo)-2-naphthol	2	3.25 ± 0.34	3.00 ± 0.18[d]
Sudan I	2	4.25 ± 0.31	3.36 ± 0.12[d]
Sudan II	2	2.90 ± 0.20	2.54 ± 0.20[d]
Sudan III	2	1.80 ± 0.08	2.28 ± 0.20[d]
Diphenols			
Catechol	30	1.98 ± 0.11	1.79 ± 0.20[e]
Resorcinol	30	1.09 ± 0.08	0.88 ± 0.04[e]
Hydroquinone	30	2.35 ± 0.19	1.92 ± 0.12[e]
tert-Butylcatechol	30	1.75 ± 0.17	1.65 ± 0.10[e]
tert-Butylresorcinol	30	0.97 ± 0.08	0.79 ± 0.08[e]
tert-Butylhydroquinone	30	2.66 ± 0.23	2.87 ± 0.38[e]
Isothiocyanate			
Benzylisothiocyanate	5	4.91 ± 0.20	3.16 ± 0.62[d]
Dithiolthiones			
1,2-Dithiol-3-thione	10	2.65 ± 0.25	2.95 ± 0.40[d]
4-Phenyl-1,2-dithiol-3-thione	30	3.53 ± 0.34	3.46 ± 0.56[d]
5-(2-Pyrazinyl)-4-methyl-1,2-dithiol-3-thione	30	1.59 ± 0.14	2.32 ± 0.34[d]

[a] Direct assay described under Materials and Methods ($N = 8$).
[b] Hepa 1c1c7 cells grown, treated, and assayed from 75-cm² plates as described by DeLong *et al.* (25).
[c] Mean value ± standard deviation.
[d] Unpublished results ($N = 4$).
[e] From Prochaska *et al.* (24).

medium, and grown for an additional 24 h. The total cellular protein of each well of one plate was determined. The wells in this plate were washed with phosphate-buffered saline, 400 μl of water was added, and the wells were sonicated. Aliquots from each well were assayed by the method of Bradford (28), with bovine serum albumin as standard. A second plate was used to determine cell number per well, and the remaining four plates were stained with crystal violet and destained as described for the 96-well plates. The stain from each well was solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol and the absorbance of the resulting solution was measured in 1.0-cm light path cuvettes at 610 nm. The average absorbance for every concentration of cells from each individual

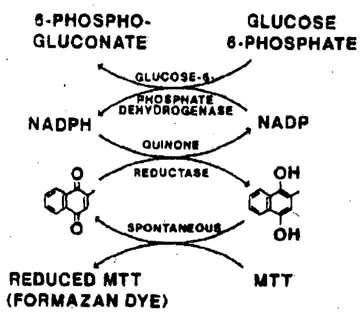

FIG. 1. Principle of the assay of quinone reductase. Glucose 6-phosphate and glucose-6-phosphate dehydrogenase continually generate NADPH, which is used by quinone reductase to transfer electrons to menadione. The menadiol reduces MTT to the blue formazan which can be measured over a broad range of wavelengths (550–640 nm). The complete reaction mixture is described under Materials and Methods. Both NADPH and menadione are regenerated, which obviates problems encountered with substrate depletion.

plate was used to determine the mean and standard error of absorbances shown in Fig. 4.

Determination of specific activities. The results of quinone reductase specific activity measurements for Table 2 are reported as the ratios of specific activities of inducer-treated microtiter wells to those of controls. The rate of MTT reduction and the crystal violet absorbances for the inducer-treated groups were compared to those of control cells grown on the same microtiter plates. The results were calculated using a spread-sheet program and the standard deviations shown in Table 2 were determined from the standard deviations of both the MTT and crystal violet assays.

RESULTS AND DISCUSSION

The assay (Fig. 1) is based on the production of a blue color when MTT is reduced nonenzymatically by menadiol that is generated enzymatically from menadione by quinone reductase. Similar systems have been used for staining quinone reductase activity in gels (29). Although the bleaching of the color of 2,6-dichloroindophenol (a substrate for quinone reductase) by reduced nicotinamide nucleotides can be followed in microtiter wells, its use is unsatisfactory in this assay system for two main reasons. First, the depletion of 2,6-dichloroindophenol results in the significant decline of reaction rate with time. Second, small errors in pipetting of the reaction mixture containing 2,6-dichloroindophenol (which has an absorbance of 1.8 to 2.0 under usual assay conditions) can result in significant variability. These errors adversely affect the reproducibility of data since only the *absolute* absorbance at 5 min rather than the absorbance *change* in each well can be conveniently measured. The use of MTT reduction avoids these difficulties since (a) the menadione concentration remains constant in the assay system because MTT reduction results in menadione regeneration and (b) the assay depends on the generation of color from absorbances that are initially negligible. Thus, all wells have negligible absorbance at zero time and the absolute ab-

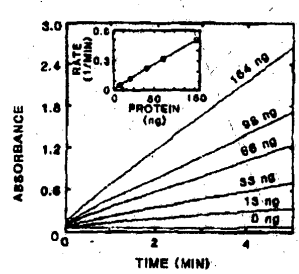

FIG. 2. Dependence of MTT reduction rate on amount of quinone reductase. Pure murine quinone reductase (30) was added in the indicated quantities and the rates of MTT reduction were recorded for 5 min at 610 nm in 1.0-cm light path cuvettes and in total volumes of 3.0 mL. The reaction mixture was identical to that used for microtiter well assays. Menadione was added to initiate the reaction. The assay was linear for 5 min for absorbance changes of up to 0.5/min. The rates obtained were proportional to the amount of enzyme added (inset; $r = 0.999$).

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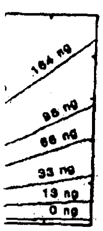

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sorbance at 5 min accurately reflects the change in absorbance during this time. The extinction coefficient of reduced MTT was found to be 11,300 M^{-1} cm^{-1} at 610 nm.

The assay utilizes an NADPH generating system that maintains a constant NADPH concentration, which can be varied as desired. Because the rate of MTT reduction by the number of Hepa 1c1c7 cells normally grown in a microtiter well is too rapid to measure conveniently if the NADPH concentration is saturating, 24 μM NADPH was chosen ($\approx \frac{1}{2} K_m$; (30)). NADH is unsuitable for this assay because MTT reduction is also promoted by other NADH-linked dehydrogenases that are neither dicoumarol inhibitable nor menadione dependent. Thus, the use of NADH results in high nonspecific rates. With NADPH, however, the menadione-independent or dicoumarol-insensitive reduction of MTT with control cells was less than 10% of the total activity obtained in the standard assay (Table 1). This slow rate of reduction of MTT (caused by nonspecific diaphorases) is almost the same in both the presence of dicoumarol and in the absence of menadione, and it is unaffected by induction of the specific NAD(P)H:quinone reductase. Because the basal rate of reduction of MTT by NADPH is low, there is no need to run parallel dicoumarol-inhibited plates during routine screening.

Since both substrates are regenerated, the absorbance is linearly proportional to the amount of added enzyme up to a rate of absorbance change of 0.5 per min (Fig. 2). The rate of absorbance change in the absence of enzyme is 0.001/min. Furthermore, the rate of MTT reduction in the assay utilized for microtiter wells is linearly related to the rate of NADH oxidation as measured in the standard menadione assay described by Prochaska and Talalay (30; Fig. 3). The addition of dicoumarol (50 μM final concentration) to the cuvette under conditions similar to those used for the microtiter assay results in virtually instantaneous inhibition of MTT reduction. The rapidity of inhibition is to be

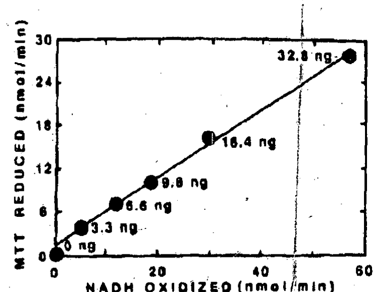


FIG. 3. Comparison of quinone reductase activity measured by the NADPH-dependent menadiol-mediated reduction of MTT with the standard quinone reductase assay. Six amounts of pure quinone reductase (30) indicated in the figure were assayed in the standard assay for quinone reductase as described by Prochaska and Talalay (30) by following the oxidation of NADH (200 μM) by menadione (50 μM) at 340 nm, as well as in the assay system utilized for microtiter wells by following the reduction of MTT by NADPH at 610 nm. Both assays were performed at 25°C in 1.0-cm light path cuvettes and in total volumes of 3.0 ml. The rates obtained by the two assays are linearly correlated ($r = 0.998$) and the MTT assay rate was 46.7% that of the standard NADH assay.

expected since the K_i value for dicoumarol is low (110 nM) and the concentration of the competing substrate (NADPH) is also low (20 μM) (30). Thus, this system provides an appropriate assay for quinone reductase.

Because we were interested in maximizing the rapidity with which the screening of inducers could be performed, we modified the method of crystal violet staining described by Drysdale *et al.* (27). This procedure has been used with great success to determine the specific activity of cytotoxic factors in the L929 murine fibroblast line, since it is a rapid, simple, and reliable method for determining cell number (27). Staining with crystal violet also appears to be well suited for the Hepa 1c1c7 murine hepatoma cell line since the degree of crystal violet absorption correlates well with cell number and total protein (Fig. 4; $r = 0.996$ and 0.997, respectively). Indeed, at exceedingly high cell densities,

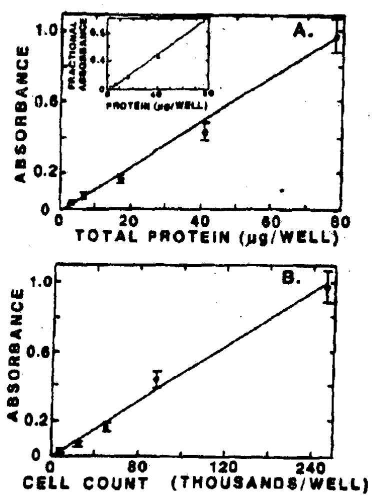


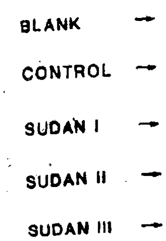
FIG. 4. Crystal violet staining correlates with total cellular protein (A) and cell number (B). Hepa 1c1c7 cells were plated at five cell densities in six identical 24-well 2-cm^2 plates and grown as described under Materials and Methods. Aliquots from one plate were used to estimate cell protein (presented as µg of protein per well) by the method of Bradford (28), and a second plate was used to determine cell number. The remaining four plates were stained, destained, and solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol. The solutions were transferred to 1.0-cm light path cuvettes and the absorbances were then determined at 610 nm. The average crystal violet absorbance for every concentration of cells from each plate was determined, from which the mean absorbances and standard errors shown in the figure were calculated ($N = 4$). The absorbances of crystal violet were linearly correlated with total protein and cell number ($r = 0.997$ and 0.996, respectively). Furthermore, the proportion of crystal violet absorbance relative to the highest absorbance was virtually the same between plates (inset).

cm^2, the crystal violet absorbance continues to correlate well with total protein, although the ratio of crystal violet absorbances to cell number increases (data not shown). Although there is some variability of the absolute absorbance of crystal violet between individual plates, the relative proportion of staining of cells of different cell densities is remarkably constant (Fig. 4, inset). Hence, we find that crystal violet staining is a suitable method for the rapid estimation of total cellular protein and/or cell number and the data from Fig. 4 can be used to derive a simple formula for estimating quinone reductase specific activity.[3] We found that over many ($N = 32$) experiments the specific activities ranged between 104 ± 3.4 and 355 ± 18.7 nmol/min/mg, and the mean ± standard deviation of the averages is 208 ± 66 nmol/min/mg. The specific activity of quinone reductase in Hepa 1c1c7 cells grown in 75-cm^2 plates and assayed in the conventional manner with menadione (50 µM) and NADH (200 µM) as substrates ranged from 213 ± 6.6 to 578 ± 81.6 nmol/min/mg of protein. The mean and standard deviation of the averages

[3] The specific activity of quinone reductase (nmol/min/mg of protein) can be estimated by using the extinction coefficient of MTT (11,300 M^{-1} cm^{-1} at 610 nm) and the measure of total cellular protein as determined by the proportionality constant calculated from the calibration curve for crystal violet. This constant (37.8 ml/mg/cm light path at 610 nm) is the slope of the line depicted in Fig. 4A multiplied by 3.0 ml (the volume in which the crystal violet stain was solubilized). Because of the orientation of the light beam relative to the microtiter well, the absorbance of a given quantity of chromophore is independent of volume; i.e., the product of the concentration and path length is a constant. In other words, for a given amount of chromophore, if the concentration is halved by the addition of solvent, the path length is doubled and the total absorbance remains unchanged. Thus, the moles of MTT reduced or the mass of protein per well can be determined from their respective absorbances, the extinction coefficient or proportionality constant, and the area of the microtiter well (0.32 cm^2). Furthermore, since both the MTT and crystal violet assays are scanned in microtiter wells of the same diameter, the specific activity calculation becomes independent of area. Therefore, specific activity can be calculated from the simple formula

$$\text{specific activity} = \frac{\text{absorbance change of MTT/min}}{\text{absorbance of crystal violet}} \times 3345 \text{ nmol/mg},$$

where 3345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT.



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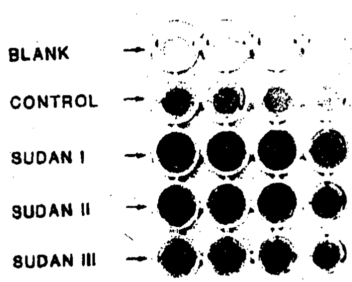

FIG. 5. Photograph showing the color (blue) that develops in the assays for quinone reductase activity of Hepa 1c1c7 cells grown in microtiter wells. The cells were grown, induced, and assayed as described under Materials and Methods. Blank wells contain no cells; control wells contain Hepa 1c1c7 cells treated with medium containing 0.1% DMSO (without inducer). Sudan I, II, and III wells contain cells that were treated with media containing the respective azo dye (2 μM) in 0.1% DMSO. All cells were grown for 24 h and then treated with inducer or DMSO for another 24 h prior to assay. Four identical wells are shown for each condition.

of 15 experiments is 357 ± 106 nmol/min/mg of protein.

The usefulness of the microtiter system in screening for inducers of quinone reductase is illustrated in Table 2 and Fig. 5. This assay accurately identified inducers and noninducers and yielded virtually the same rank order of induction as did experiments with cells grown on 75-cm^2 plates and assayed in the conventional manner. For example, we have reported that resorcinol and its substituted congeners were inactive as inducers of quinone reductase, whereas catechols and hydroquinones could significantly elevate levels of quinone reductase in the Hepa 1c1c7 cell line (24). The same patterns were observed with the diphenols tested in the direct assay system (Table 2). Furthermore, the rank order of induction potency of azo dyes tested in the direct assay is the same as in the conventional assay system. Figure 5 demonstrates that the degree of quinone reductase induction can be detected without the assistance of a microtiter scanner. Scanning of the absorbances for the experiment shown in Table 2 required less time than did harvesting of cells from the equivalent number of 75-cm^2 plates. Data processing can be further simplified by linking the scanner to a personal computer. We conclude that the direct assay of quinone reductase from cells grown in microtiter wells may facilitate the identification and isolation of novel inducers of chemoprotective enzymes such as quinone reductase.

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Cancer preventive properties of varieties of *Brassica oleracea:* a review[1–3]

Christopher WW Beecher

ABSTRACT Cabbage, broccoli, Brussels sprouts, and other members of the genus *Brassica* have been widely regarded as potentially cancer preventative. This view is often based on both experimental testing of crude extracts and epidemiological data. The experimental evidence that provides support for this possibility is reviewed for the commonly consumed varieties of *Brassica oleracea.* In a majority of cases the biological activities seen in testing crude extracts may be directly related to specific chemicals that have been reported to be isolated from one of these closely related species, thus the chemical evidence further supports the data from testing extracts and epidemiology. *Am J Clin Nutr* 1994;59(suppl):1166S–70S.

KEY WORDS *Brassica,* Brassicaceae, mutagen, antimutagen, cancer, prevention, vegetables, chemoprevention

Introduction

Although most botanists would hardly agree that "A rose may be a rose by any other name" there would be substantial agreement that a cabbage and a cauliflower may be quite the same. These vegetables, and other closely related members of the Brassicaceae family, have received widespread notice recently as public figures have disavowed their consumption and scientists have upheld them as exemplary of medicinaly significant foods. Thus, in this article we review all of the experimental evidence that suggests that there may be a cancer preventive benefit from consumption of members of these closely related and commonly consumed vegetables (1, 2). Furthermore, in view of the extensive data (3, 4) that exist for these vegetables, we will restrict ourselves to those vegetables commonly classified as subvarieties of the species *Brassica oleracea* (Table 1).

From the outset it must be realized that the published experimental data come from two different types of experimental protocols. In the first type, evidence is published that concerns tests conducted on the whole food (or from crude extracts). In the second type, tests are conducted on specific chemical compounds that have been isolated from these foods. Specifically, we will cross-correlate these two bodies of data so that, whenever possible, the specific compounds that may be responsible for an observation seen in testing a crude extract are identified. It is worth noting that this information is often not available in the original article and lends credence to the initial observation.

It is our intention to provide support for observations made on crude extract and identify those areas in which the biologically active chemical species for a given observation may not yet be identified. Although various aspects of the chemistry (5), pharmacology (6, 7), biology (8–10), and general concepts of cancer chemoprevention (11–13) have been reviewed separately, we will provide an overview approach that demonstrates the overlap between these various areas. Furthermore, it is important to recognize that many clinical trials are currently underway, (14) which, in preliminary reports, lend credence to the cancer preventative approaches (15, 16).

Relevant biological activities

The etiology of cancer follows no single track but rather is the result of an accumulation of diverse events that lead to a common endpoint, namely the uncontrolled growth of a normally quiescent cell. Nevertheless, there are generally recognized to be many common stages to the development of cancer. These stages (Fig 1) include an initial insult (or mutation) to the genetic material often delivered by a mutagen or other chemical agent but may also be inherited or possibly viral in origin. A cell that has received such an insult is said to be initiated. An initiated cell will still be quiescent and not manifest its altered phenotype until it is promoted. The promotional act may similarly take multiple forms but it fundamentally involves achieving a physiological state that signals the altered DNA to be read. Where the altered message leads to an unquenchable cycle of cellular division, the cell is considered cancerous. This aberrant equilibrium, where the cell cannot reset itself, will become a tumor if it cannot regain a "normal" or self-restrained equilibrium.

Cancer chemotherapeutic agents are directed against cancerous or fully promoted cells and seek to selectively kill the cell based on some aspect of its aberrant biochemical equilibrium. As such, all current cancer treatment is based on compounds that are toxic. An ideal cancer chemotherapeutic agent would be toxic only to cancer cells but the reality is that such specificity has not yet been acheived. Although this is clearly a suitable course when the fatality of the disease is considered, the approach to cancer

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TABLE 1
The most commonly consumed members of the genus *Brassica*

Species	Variety	Common name
Brassica campestris		Field mustard
Brassica chinensis		Bok choy
Brassica juncea		Mustard greens
Brassica napus	var *napabrassica*	Rutabaga
Brassica nigra		Black mustard
Brassica oleracea	var *acephala*	Collards
Brassica oleracea	var *acephala*	Kale
Brassica oleracea	var *botrytis*	Broccoli
Brassica oleracea	var *botrytis*	Cauliflower
Brassica oleracea	var *capitata*	Cabbage
Brassica oleracea	var *gemmifera*	Brussels sprouts
Brassica oleracea	var *gorgylodes*	Kohlrabi
Brassica pekinensis	var *capitata*	Cabbage (Chinese)
Brassica rapa	var *rapifera*	Turnip
Brassica rapa	var *japonica*	Red turnip

chemoprevention must be based on a very different strategy. In view of the fact that such agents must be used prophylactically, they must exhibit few, if any, side effects and must have virtually no toxicity. In addition to these stringent requirements, it needs to be recognized that any compound that is to be considered as a cancer chemopreventive agent may also exhibit a suitable spectrum of biological activity.

In cancer chemoprevention the aim is to reduce the number of initiated cells, inhibit the promotion of initiated cells, or even reverse the promotion itself. Furthermore, each of these broad categories has many strategies that may be useful to cancer prevention. First, there are strategies that aim to reduce the initiation rate. The agents here are classified as antimutagens, desmutagens, inhibitors of enzymes that activate procarcinogens, or agents that stimulate the metabolization of mutagens to less harmful metabolites. Also included here are antioxidants because a portion of the genetic damage is likely the result of free radical damage (17–21). Second, there are strategies that aim to reverse or inhibit the promotion stages. Biological activities that act at this stage may act specifically on the promoted cell to cause it to redifferentiate and hence regain control of its own division or they may act at any of the points in one of the secondary messenger (or the related oncogene) systems that are frequently implicated as destabilizing agents. In the same light, the biological consequences of low-level inflammation or constant low-level estrogenic stimulation are similarly considered destabilizing (22, 23) and hence targets for chemopreventive approaches.

As we consider such an etiology, we can associate specific bioassays that have been described in the literature in relation to one or more of these points. Thus, as a basis for this article, we have undertaken to review the reports of relevant biological activities for *Brassica oleracea* varieties. They will be organized as discussed above. With respect to initiation stages, the majority of published literature in this area may be divided crudely into two broad groupings, namely reports of an anti- (or des-) mutagenic activity and reports of stimulation of a detoxification mechanism. With respect to antipromotion activity, there is a single report that suggests that this mechanism may play a role in *Brassica's* cancer preventive potential. However, there are many reports that may not be classified as either of these stages but

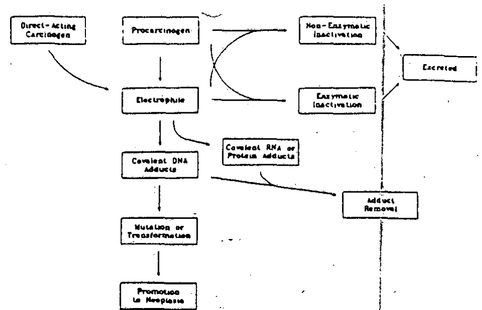

FIG 1. The etiology of cancer. The steps shown are those generally assumed in the development of cancer.

rather are of such a general nature that no specific mechanism of protection may be ascribed to them.

Antimutagenic activities

The ability of a crude extract of a *Brassica* variety to reduce the effect of a mutagen (either as a desmutagenic agent or as an antimutagenic agent) has been reported no less than eight times. In all of the cases in which a mechanism can be discerned it appears that, although the term antimutagen is used routinely, these are most likely all cases of desmutagenicity. These reports are summarized in **Table 2**.

The major bulk of the reports concern ability of a protein, termed the desmutagenic factor, to inhibit various mutagens in Ames-type assays. This factor, first described by Kada *et al* (24), was later characterized (25) and patented (26) by Morita et al as a heat-labile protein with a molecular weight of ≈53 kDa, which contained a prosthetic group with a heme-like chromophore. This protein was shown active against tryptophan pyrolysates (24), ethidium bromide (25), 2-aminoanthracene (25), autooxidized linolenic acid (27), and pyrolysates for other amino acids (28).

TABLE 2
Summary of antimutagenic results

Plant extracted	Mutagen	Percent reduction
		%
Cauliflower	Nitrate + methylurea	78
Cauliflower	Nitrate + aminopyrine	57
Cabbage	Nitrate + sorbic acid	Moderate (not calculable)
Cauliflower	Nitrate + sorbic acid	Moderate (not calculable)
Cabbage	Tryptophan pyrolysate	97
Broccoli	Tryptophan pyrolysate-1	97
Broccoli	Tryptophan pyrolysate-2	81
Broccoli	Ethidium bromide	92
Broccoli	2-Aminoanthracene	84
Broccoli	AF-2	0
Broccoli	Oxidized linolenic acid	82
Cabbage	Oxidized linolenic acid'	76
Red cabbage	Oxidized linolenic acid	81
Cauliflower'	Oxidized linolenic acid	76
Cabbage	Tryptophan pyrolysate-2	35

In their 1980 paper, Yamaguchi et al (27) demonstrated a striking correlation between the desmutagenic activity of the extracts and their peroxidase activity and further demonstrated that the peroxidase activity required a cofactor. This activity was later confirmed in the purified protein by Morita et al (25), who did not note the need for the cofactor. The signal characteristic to the desmutagenic factor has always been the fact that it is both heat labile and is inactivated by digestion with a proteinase. With this in mind, some workers (29) have pointed out that after heat treatment some crude extracts of *Brassica* extracts still exhibit residual activity, suggesting the presence of other antimutagenic components. Munzer (30) demonstrates that some antimutagenic activity acts by stimulating native detoxification systems in *Salmonella typhimurium* and thus some of these other agents are also desmutagenic.

The identity of the other antimutagenic agents has been the focus of other researchers. Two groups (31, 32) have reported that extracts of cauliflower and cabbage, respectively, interfere with the production of mutagens by nitrosation. There is considerable agreement that the active agents include ascorbic acid, cysteine, or other compounds acting as reducing agents. This is actually demonstrated by Osawa et al (32), who show that the ascorbic acid is responsible for the chemical reduction of the 1,2-dinitro-2-methyl pyrrole, the mutagenic nitrosation product of sorbic acid, to the nonmutagenic compound 1-nitro-2-methyl-4-amino pyrrole. Barale et al (31) show that ascorbic acid and some phenolic compounds can duplicate the activity seen in the crude extract. On the other hand, Lawson et al (33) have identified four specific compounds isolated from savoy chieftain cabbage that demonstrated antimutagenic activity against specific mutagens, *N*-methyl-*N*-nitrosourea (NMU) and 2-aminoanthracene (2-AA). These compounds, β-sitosterol, pheophytin-a, nonacosane, and nonacosanone, are notable because they are likely to be present in a majority of plants. These authors also demonstrate that commercial chlorophyll, the biological precursor to pheophytin-a, is strongly antimutagenic. These compounds were shown to present different activity profiles against the NMU and 2-AA; therefore, the authors argue that these compounds were achieving their antimutagenicity through more than one biological mechanism.

Stimulation of detoxification mechanisms

As noted briefly above, Munzer (30) noted that the antimutagenic activity of many vegetables, including cabbage, Brussels sprouts, and kohlrabi, was in stimulating the S-9 mix normally used to metabolize and sometimes activate mutagens. This observation serves to bridge the antimutagenic potential discussed above and the large body of data that makes it clear that in animals there is a strong stimulation of many of the native detoxification systems by extracts of various *Brassica* species. Although this attribute has been fairly widely discussed recently, because of the articles published by Talalay's group (34, 35), it is important to note that this area has a long and honorable background. Furthermore, although the Talalay articles do demonstrate a selectivity in the induction of phase-2 enzymes that has not previously reported, the ability of members of the Brassicaceae family to stimulate a broad spectrum of enzyme systems has been widely reported.

The earliest work on the induction of these enzyme systems was actually an attempt by Wattenberg (36) to explain variations in baseline aryl hydrocarbon hydroxylase concentrations in different rat colonies. The variation ultimately was ascribed to the presence of alfalfa as an occasional component in rat chow. This observation was followed by an examination of the ability of many foods to stimulate this enzyme. Wattenberg and his group demonstrated that many members of the Brassicaceae family were also active in this regard (37) and, furthermore, the active compounds were readily identified as indole-3-carbinol, 3,3'-diindolymethane, and indole-3-acetonitrile, which stimulated 50-fold, 20-fold, and 6-fold increases, respectively, in enzyme activities in the livers of rats that consumed augmented basal chow. In subsequent papers they demonstrated that the ability of intestinal enzymes to detoxify many xenobiotic compounds, including the indoles noted above (38), correlated to Brussels sprouts or cabbage consumption in rats (39) and in humans (40). The enzyme systems involved included many mixed-function oxidases, such as phenacetin O-dealykylase, 7-ethoxycoumarin O-dealkylase, hexobarbital hydroxylase, and benzo[a]pyrene hydroxylase. A direct correlation was later established between the induction of these activities and the concentration of these compounds by McDanell et al (41, 42). These later studies also demonstrated that the various active compounds had differing abilities to stimulate enzymes in different organs of the body. They note for instance that the ascorbic acid conjugate of indole-3-carbinol is the most active compound in stimulating the mixed-function oxidase populations of the gut whereas indole-3-carbinol, of the compounds tested, was the strongest inducer of the liver enzymes. Tanaka et al (43) demonstrated recently the ability of indole-3-carbinol to inhibit tongue carcinogenesis induced with 4-nitroquinoline-1-oxide.

Meanwhile, working in a parallel vein, Salbe and Bjeldanes (44) not only confirmed the earlier results of the Wattenberg group but also demonstrated that the enzyme glutathione-*S*-transferase was also strongly induced by Brussels sprouts. This enzyme, unlike those discussed earlier, is not a P-450 type enzyme but represents rather a phase-2 detoxification system that acts to conjugate and clear toxicants from the system. The significance of this difference cannot be understated. For most of the P-450 type enzymes, their ability to detoxify many mutagens must always be balanced by their ability to activate other mutagens (45). For glutathione-*S*-transferase, there are no such drawbacks, rather, as this group has shown (46), an increase in this enzyme alone directly resulted in an 87% reduction in the binding of aflatoxin to hepatic DNA in vivo. A wide spectrum of compounds (47, 48) including the glucosinolates, such as sinigrin and progoitrin, and their derivates, such as allyl isothiocyanate, goitrin, indole-3-carbinol, and indole-acetonitrile, induce glutathione-*S*-transferase. In other systems it is induced even more strongly by xanthotoxin and some flavanoids (49).

Other relevant reports

There are some reports in which no mechanism can be easily ascribed to the results or that do not fit into either of the above two categories. These reports are nonetheless potentially significant with respect to the ability of brassicaceous plants to be cancer chemopreventive. The first of these concerns a study conducted by Bresnick et al (50) in which rats were fed a controlled fat diet with and without cabbage. There was found to be a statistically significant reduction in the rate of chemically induced

breast tumors in the rats with cabbage in their diet. This effect was not seen in rats on a high-fat diet. It is of interest to note that the experimental design allowed for the consumption of cabbage only after the initiation event, administration of MNU, thus indicating a potential antipromotion effect. This possibility is also suggested by a report from Koshimizu et al (51), who use the inhibition of Epstein-Barr virus induction as an indication of antipromotion activity. In their assay an extract of cauliflower is very strongly active in inhibiting the normal promotion event. In neither of these publications is it possible to ascribe a specific compound to the activity observed.

Finally, note that protease inhibitors have been associated with carcinogenesis inhibition (52, 53), so the relevance of a strong trypsin inhibitor from the seed of kale (*Brassica oleracea* var *sabellica*) may be relevant (54). The presence of this agent in other parts of the plant (much less in other varieties) or its ability to overcome problems of absorption and transport are totally unknown.

Summary

It may at first seem surprising that so many biological activities have been demonstrated for plants as commonly consumed as these. Yet reflection on the complex chemical nature of most plants suggests that there may be more biological potential in all of them than we would expect from something that is generally considered to be biologically neutral. Furthermore, although some of these reports have been in humans, the majority are in vitro results whose bearing on their effect on humans is very much an open question. The work of McDanell et al (41, 42) clearly demonstrates the importance of transport and the variable ability of different metabolites of even the same compound to affect different organs. A report by Birt et al (55) amplifies this by demonstrating that although the effect of cabbage is beneficial in some cases it may act to increase tumorigenicity in other model systems (or cancer types).

We have presented a case that strongly implies that the cancer preventive potential of many members of the Brassicaceae family is strong, yet it must always be stressed that to understand the relevance of these reports on the human condition, many further studies need to be done to specifically address questions of the stability, bioavailability, transport, and metabolism. The additive or even synergistic effects of these compounds are unknown. The additional effects of normal food preparation procedures present another factor that is yet largely unexplored with respect to the cancer preventive properties. In brief, there is much exciting potential in the cancer preventive properties and yet there is, as of this writing, no absolute statement that can be made concerning the ability of these foods to directly alter the course of carcinogenesis.

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Chemical and molecular regulation of enzymes that detoxify carcinogens

(chemoprotection/electrophiles/quinone reductase/transient gene expression/phase 2 enzymes)

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ABSTRACT **Inductions of detoxication (phase 2) enzymes, such as glutathione transferases and NAD(P)H:(quinone-acceptor) oxidoreductase, are a major mechanism for protecting animals and their cells against the toxic and neoplastic effects of carcinogens. These inductions result from enhanced transcription, and they are evoked by diverse chemical agents: oxidizable diphenols and phenylenediamines; Michael reaction acceptors; organic isothiocyanates; other electrophiles—e.g., alkyl and aryl halides; metal ions—e.g., $HgCl_2$ and $CdCl_2$; trivalent arsenic derivatives; vicinal dimercaptans; organic hydroperoxides and hydrogen peroxide; and 1,2-dithiole-3-thiones. The molecular mechanisms of these inductions were analyzed with the help of a construct containing a 41-bp enhancer element derived from the 5′ upstream region of the mouse liver glutathione transferase Ya subunit gene ligated to the 5′ end of the isolated promoter region of this gene, and inserted into a plasmid containing a human growth hormone reporter gene. When this construct was transfected into Hep G2 human hepatoma cells, the concentrations of 28 compounds (from the above classes) required to double growth hormone production, and the concentrations required to double quinone reductase specific activities in Hepa 1c1c7 cells, spanned a range of four orders of magnitude but were closely linearly correlated. Six compounds tested were inactive in both systems. A 26-bp subregion of the above enhancer oligonucleotide (containing the two tandem "AP-1-like" sites but lacking the preceding ETS protein binding sequence) was considerably less responsive to the same inducers. We conclude that the 41-bp enhancer element mediates most, if not all, of the phase 2 enzyme inducer activity of all of these widely different classes of compounds.**

Elevation of the activities of phase 2 detoxication enzymes† of cells provides protection against neoplasia (6). This paper analyzes the chemical and molecular specificity of the regulation of phase 2 enzymes, as part of our efforts to develop novel approaches to chemoprotection against cancer. Phase 2 enzymes, which are widely distributed in mammalian cells and tissues, include the following: glutathione (GSH) transferases, which conjugate mostly hydrophobic electrophiles with GSH; QR, which promotes obligatory two-electron reductions of quinones, preventing their participation in oxidative cycling and the depletion of intracellular GSH; epoxide hydrolase, which inactivates epoxides and arene oxides by hydration to diols; and UDP-glucuronosyltransferases, which conjugate xenobiotics with glucuronic acid, thus facilitating their excretion. The induction of these enzymes is accompanied by elevations of intracellular GSH levels which augment cellular protection (7–11).

Induction of phase 2 enzymes is evoked by an extraordinary variety of chemical agents, including Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, vicinal dimercaptans, heavy metals, arsenicals, and others (12–14). With few exceptions these agents are electrophiles (or can be converted to electrophiles by metabolism), and accordingly, many of these inducers are substrates for glutathione transferases (13).

The molecular basis of the regulation of phase 2 enzyme inductions has been analyzed by deletions of the 5′ upstream regulatory regions of glutathione transferase Ya subunit genes and QR genes after transfection of cells with chloramphenicol acetyltransferase (CAT) constructs (3, 15–17). The sequences of the upstream enhancer elements of the mouse and rat liver glutathione transferase Ya subunit genes that respond to the few inducers tested are very similar and have been termed the electrophile-responsive element (EpRE) (18) and the antioxidant-responsive element (ARE) (19), respectively. These elements (Fig. 1) are contained within a 41-nt segment located between base pairs -754 and -714 in the mouse, and -722 and -682 in the rat Ya gene. The critical DNA sequences responsive to monofunctional inducers appear to be the TGACAT/AT/AGC regions, which resemble AP-1 binding sites (20). Similar enhancer sequences have also been identified in the upstream regulatory regions of the rat and human QR genes (3, 4, 17). We show that all the chemical inducers of phase 2 enzymes that we tested stimulate expression of a reporter gene through this 41-bp enhancer element.

MATERIALS AND METHODS

Cell Culture. For the growth hormone (GH) transient gene expression assays the cells were grown in Eagle's minimal

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Abbreviations and definitions: AP-1, a family of transcriptional activator DNA-binding proteins that bind to the consensus sequence TGAC/GTC/AA; CAT, chloramphenicol acetyltransferase; CD_{GH}, concentration of an inducer that doubles the production of growth hormone in a transient gene expression assay; CD_{QR}, concentration of an inducer that doubles the quinone reductase specific activity in Hepa 1c1c7 cells; DMSO, dimethyl sulfoxide; ETS, a family of transcriptional activator DNA-binding proteins; GH, growth hormone; QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; sulforaphane, 1-isothiocyanato-(4*R*)-(methylsulfinyl)butane [CH_3—SO—$(CH_2)_4$—NCS].

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†Two broad classes of enzymes metabolize xenobiotics: (*i*) phase 1 enzymes, which functionalize molecules by introducing hydroxyl or epoxide groups and (*ii*) phase 2 enzymes (1), which detoxify either by conjugating these functionalized molecules with endogenous ligands (e.g., glutathione), thus facilitating their excretion, or by destroying their reactive centers by other reactions [e.g., hydrolysis of epoxides by epoxide hydrolase or reduction of quinones by quinone reductase (QR)]. Reasons for considering QR a phase 2 enzyme are presented elsewhere (2–4). Inducers of enzymes of xenobiotic metabolism belong to two families (5): (*i*) bifunctional inducers, which bind to the aryl hydrocarbon (Ah) receptor and induce certain phase 1 enzymes and phase 2 enzymes and (*ii*) monofunctional inducers, which induce phase 2 enzymes independently of the Ah receptor.

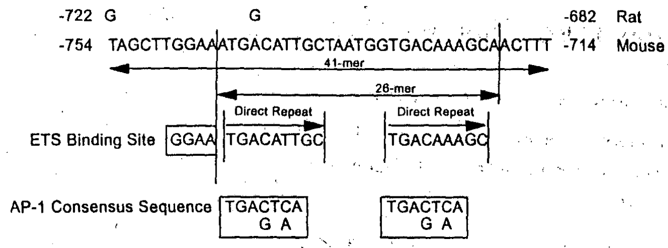


FIG. 1. Highly homologous 41-bp enhancer sequences from the upstream region of the mouse and rat glutathione transferase Ya subunit genes, representing bases -682 to -722 (rat) and -714 to -754 (mouse) from the origins of replication. Two "AP-1-like" regions are present with a core ETS protein binding site located next to the first AP-1 site in the mouse sequence.

essential medium supplemented with Earle's balanced salt solution, nonessential amino acids, sodium pyruvate, glutamine, and 10% fetal calf serum. All cells were maintained in a humidified atmosphere of 5–7% CO_2 at 37°C. Cell lines were free from mycoplasma.

Compounds. Most inducers were obtained commercially. Racemic sulforaphane was synthesized by C.-G. Cho and G. H. Posner (21).

Plasmids and Their Constructions. The plasmids 41YaCAT and -187YaCAT (15) and RSVgal were gifts of Violet Daniel (The Weizmann Institute of Science, Rehovot, Israel). The plasmid pCH110 was obtained from Pharmacia. p41YaCAT contains a portion of the upstream sequence of the mouse glutathione transferase Ya gene from -1594 to the *Bgl* II site at -1272 where the 41-bp EpRE is present in the reverse orientation (20). The EpRE is linked directly to the intact *Bgl* II site at nucleotide -187 in the upstream region. p284YaGH was prepared by ligating a 284-nt fragment (representing the sequence from -186 to +98) containing the mouse glutathione transferase Ya minimal promoter (as in -187 YaCAT) region into the *Bam*HI site of plasmid p0GH (22). The 284-nt fragment was generated by PCR from the plasmid 41YaCAT by use of the primers 5′-GGC TTC ACT CCA TCT AGA AAG GG-3′ and 5′-TTG CAG TGC TGC AGA CCT GGG AA-3′. The fragment was gel purified, its ends were blunted, and *Bam*HI linkers were added. It was then digested with *Bam*HI and *Bgl* II to generate two fragments, the smaller, 284-nt, fragment containing 186 nucleotides of the upstream region, the first exon, and 56 nucleotides of the first intron of the mouse glutathione transferase Ya gene. The plasmid p26-284GH was prepared by first ligating the oligonucleotide 5′-agc ttA TGA CAT TGC TAA TGG TGA CAA AGC Ag-3′ (lowercase letters indicating restriction overhangs) and its complement 5′-gat ccT GCT TTG TCA CCA TTA GCA ATG TCA Ta-3′ into p0GH (which had been cleaved with *Hin*dIII and *Bam*HI) to provide p26GH. The 284-nt fragment containing the glutathione transferase Ya minimal promoter was then inserted into the *Bam*HI site of the plasmid p26GH. The plasmid p41-284GH was prepared by ligating the oligonucleotide 5′-agc tTA GCT TGG AAA TGA CAT TGC TAA TGG TGA CAA AGC AAC TTT g-3′ and its complementary oligonucleotide 5′-tcg acA AAG TTG CTT TGT CAC CAT TAG CAA TGT CAT TTC CAA GCT A-3′ into the *Hin*dIII and *Sal* I sites of p284GH. The structures of all DNA constructs were confirmed by automated sequencing.

Transfections and Transient Gene Expression Assays. Transfections were performed by the calcium phosphate method (23). Briefly, the cells were plated at a density of 3.5 × 10^6 (Hepa 1c1c7) or 7 × 10^6 (Hep G2) in 10-cm plates and medium was replaced after 14–16 hr. After a further 3 hr, the transfection mixture containing 20 μg of the specified GH construct and 12 μg of the β-galactosidase construct (RSVgal for Hep G2 and pCH110 for Hepa 1c1c7) was added. Five hours later the cells were shocked with 15% (wt/vol) glycerol for 2 min and then allowed to recover for 16–18 hr. The cells from each 10-cm plate were trypsinized and pooled. One-quarter of the cells from each plate were replated onto a 10-cm dish for β-galactosidase assay, and the remaining cells were distributed among the wells of three 24-well plates containing 1.5 ml of medium per well. After 3–4 hr the cells were treated with three or more concentrations of inducers dissolved in either dimethyl sulfoxide (DMSO) or water (arsenicals and metal salts). A final concentration of 0.2% (vol/vol) DMSO was present in all assays. After a further 48 hr, 100 μl of medium was removed from each duplicate well and assayed for GH (Allégro HGH Transient Gene Expression Assay Kit; Nichols Institute, San Juan Capistrano, CA). CAT and β-galactosidase assays were performed (23), and viability was determined by staining with crystal violet (24).

Standardization of GH Gene Expression Assay. Basal GH secretions in six independent transfections with p41-284GH in Hep G2 cells were as follows (ng of GH secreted per ml of medium in 48 hr; means of n replicates ± the coefficient of variation): 2.20 ± 2.3% (n = 6); 2.67 ± 6.9% (n = 6); 2.80 ± 5.2% (n = 6); 3.27 ± 5.4% (n = 5); 4.93 ± 12.5% (n = 4); and 6.54 ± 4% (n = 4). The mean GH production in these six transfections was 3.92 ng/ml with an uncorrected interassay coefficient of variation of ±45.7%; after normalization for transfection efficiency by β-galactosidase measurements and for cell number (by staining with crystal violet), the interassay coefficient of variation decreased to ±17%. Similar results were obtained in transfections of Hepa 1c1c7 cells. Before transfection, neither Hep G2 nor Hepa 1c1c7 cells expressed detectable GH. Furthermore, GH added to the assay systems (0–10 ng/ml; n = 6) was recovered quantitatively from the medium. GH addition did not alter the expression of GH by Hep G2 cells transfected with p41-284GH.

Comparison of Human GH and CAT Measurements. DNA elements involved in transcriptional regulation of phase 2 enzymes were previously identified by use of constructs containing the CAT reporter gene (3, 4, 15–17). To perform large numbers of assays rapidly and reproducibly, we chose the human GH gene as reporter (22) because the GH radioimmunoassay is simple, extremely sensitive, and highly quantitative. Transfection of cells in a single 10-cm culture plate permits more than 100 measurements and avoids problems associated with differences in transfection efficiencies. To verify that GH and CAT assays gave directly comparable results with our specific enhancer elements, we showed that GH and the CAT assays performed in both Hepa 1c1c7 and Hep G2 cells transfected with the p41YaCAT and p41-284GH plasmids and treated with 2,3-dimercapto-1-propanol gave parallel inductions (Table 1). In both cell lines, however, the GH assay was much more sensitive and the inductive response range (expressed as treated-to-untreated ratios) was much higher in the GH assay than in the CAT assay. Similar response patterns were observed with several other chemically unrelated inducers such as sulforaphane (data not shown). The expression of GH by Hepa 1c1c7 and Hep G2 cells transfected with the enhancerless but promoter-containing plasmids (187YaCAT and p284GH) was not increased by any of the inducers.

Table 1. Responses to 2,3-dimercapto-1-propanol of CAT and human GH transient gene expression assays in Hepa 1c1c7 and Hep G2 cells

2,3-Dimercapto-1-propanol, μM	Response ratio (treated/untreated) of cells CAT Hepa 1c1c7	CAT Hep G2	GH Hepa 1c1c7	GH Hep G2
25	1.6	1.3	2.8	2.7
50	2.5	2.6	5.0	6.9
100	3.5	4.7	6.9	18.5

The cells were transfected with the CAT reporter p41YaCAT or the GH reporter p41-284GH.

We conclude that the GH transient gene expression assay in Hep G2 cells is a highly sensitive, quantitative, and reproducible measure of transcriptional regulation and that the results obtained parallel those of CAT assays.

Measurement of Potency for Induction of QR. The inducer potency of all compounds was determined with Hepa 1c1c7 cells grown in 96-well microtiter plates (24, 25). The inducers were added in either DMSO or water. A final concentration of 0.2% DMSO was present in all wells. The CD_{QR} (concentration required to double QR specific activity) values shown in Table 2 are lower than those reported previously (12), probably due to minor modifications [use of fetal calf serum treated with charcoal (1 g/100 ml) for 90 min at 55°C, and the inclusion of 0.2% DMSO in all assays].

RESULTS AND DISCUSSION

Comparison of Efficiencies of Inducer Responses of Plasmids p26-284GH and p41-284GH in Transient Gene Expression Assays. Prior studies of the mouse enhancer sequence used the entire 41-nt segment containing both AP-1-like sites and additional flanking sequences (Fig. 1). To determine whether the two AP-1 sites are sufficient for maximal induction, we compared the expression of GH by the complete construct p41-284GH and by p26-284GH, which contains both of the AP-1-like sites but lacks 10 of the 5′ base pairs and 5 of the 3′ base pairs of the 41-mer (Fig. 1) originally identified to contain the enhancer element in the mouse and rat upstream regions (16, 18, 26, 27). GH expression was measured with a series of concentrations of the following inducers (for structures, see Table 2): 1-nitro-1-cylcohexene (**1**), *trans*-4-phenylbut-3-en-2-one (**10**), *tert*-butylhydroquinone (**14**), sulforaphane (**15**), 2,3-dimercapto-1-propanol (**21**), phenylarsine oxide (**26**), sodium arsenite (**27**), mercuric chloride (**28**), phenylmercuric chloride (**31**), 1,2-dithiole-3-thione (**33**), and β-naphthoflavone (**34**) (Fig. 2).

The basal levels of GH production by both plasmids were essentially identical when corrected for cell number and transfection efficiency. All of these compounds produced concentration-dependent inductions of GH synthesis. Surprisingly, the maximal elevations of GH produced by these compounds in cells transfected with p26-284GH were low compared with experiments with p41-284GH (Fig. 2). However, the results obtained with p26-284GH were comparable to those observed by us (data not shown) and others with similar enhancer sequences in CAT assays (19). Thus, maximal inductions obtained with p26-284GH were 3.5-fold with 60 μM *trans*-4-phenylbut-3-en-2-one and 100 μM 1,2-dithiole-3-thione. The absolute induction ratios obtained with the plasmid containing the larger insert were dramatically higher; the highest induction ratios were 24.6-fold for 60 μM *tert*-butylhydroquinone and 22.5-fold for 6 μM sulforaphane. All compounds tested showed this difference in induction ratios for the two constructs, but the effects with phenylarsine

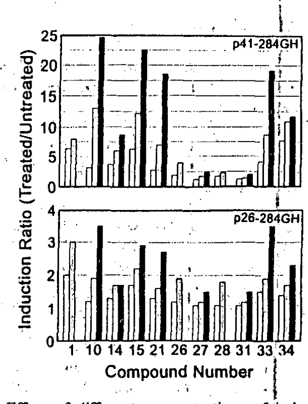


FIG. 2. Effect of different concentrations of inducers on GH production in Hep G2 cells transfected with p41-284GH (*Upper*) or p26-284GH plasmids (*Lower*). The compounds are numbered as in Table 2 and their concentrations (μM) were as follows: **1**, 1-nitro-1-cyclohexene (2.5, 5.0); **10**, *trans*-4-phenylbut-3-en-2-one (20, 40, 60); **14**, *tert*-butylhydroquinone (20, 40, 60); **15**, sulforaphane (1.5, 3.0, 6.0); **21**, 2,3-dimercapto-1-propanol (25, 50, 100); **26**, phenylarsine oxide (0.05, 0.10); **27**, sodium arsenite (2.5, 5.0, 10.0); **28**, mercuric chloride (1.25, 2.5); **31**, phenylmercuric chloride (0.5, 1.0, 2.0); **33**, 1,2-dithiole-3-thione (25, 50, 100); **34**, β-naphthoflavone (0.5, 1.0, 2.0). Open bars, low concentration; shaded bars, double the low concentration; solid bars, high concentration.

oxide, sodium arsenite, $HgCl_2$, and phenylmercuric chloride were smaller (Fig. 2). There were also large differences in the responses to inducers when p41-284GH and p26-284GH were transfected into Hepa 1c1c7 cells, although the magnitudes of induction ratios in this cell line were somewhat smaller.

In similar experiments with the rat enhancer sequence, Rushmore *et al.* (19) obtained only a 2- to 2.5-fold enhancement of CAT expression. In contrast, Friling *et al.* (18), using the 41-bp mouse enhancer sequence and the same inducers, obtained a 5- to 6-fold elevation in CAT activity, which is in accord with our results (Fig. 2). The responses of the mouse and rat enhancer sequences to inducers may differ because the 5′ region of the mouse 41-bp enhancer contains the core ETS protein DNA-binding sequence GGAA (28) near the first AP-1-like site. Adjacent ETS and AP-1 sites are known to confer dramatic synergism on gene expression (29). The rat gene lacks the first AP-1-like site, because the critical A of the AP-1 consensus is replaced by G (Fig. 1). Whether the

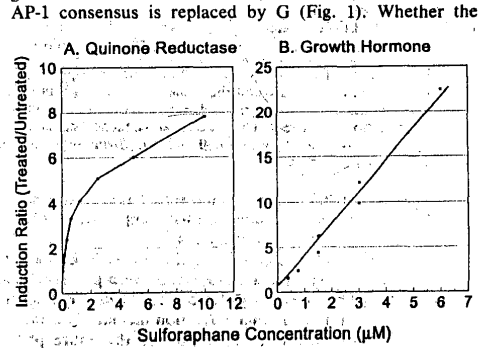
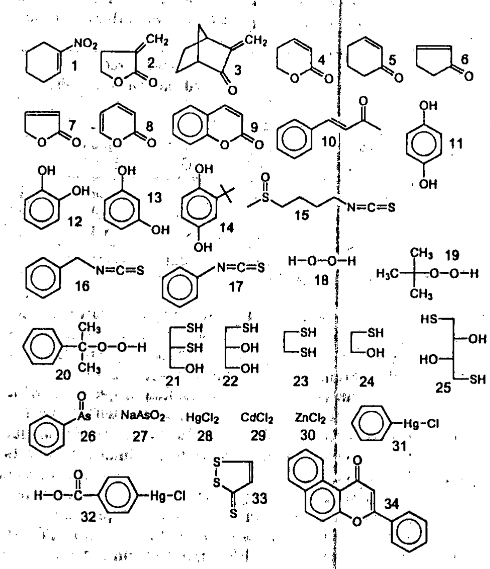


FIG. 3. Effect of increasing concentrations of sulforaphane on QR specific activity (*A*) and GH production (*B*). *B* includes data from two independent transfections, normalized for transfection efficiency.

Table 2. Potencies of inducers in enhancing GH production in Hep G2 cells transfected with p41-284GH and in elevating QR activity in Hepa 1c1c7 cells

Inducer No.	Inducer Name	CD_{GH}, μM	Rank order	CD_{QR}, μM	Rank order
Michael reaction acceptors					
1	1-Nitro-1-cyclohexene	0.98	5	0.46	5
2	2-Methylene-4-butyrolactone	2.4	8	4.5	12
3	3-Methylene-2-norbornanone	3.1	9	1.5	9
4	5,6-Dihydro-2*H*-pyran-2-one	8.8	12	6.7	16
5	1-Cyclohexen-2-one	15	17	9.1	17
6	1-Cyclopenten-2-one	80	25	32	22
7	2(5*H*)-Furanone	240	28	36	23
8	2*H*-Pyran-2-one	In	29	In	29
9	Coumarin	In	29	In	29
10	*trans*-4-Phenylbut-3-en-2-one	16	20	15	20
Diphenols and quinones					
11	Hydroquinone	12	16	5.3	14
12	Catechol	8.5	11	4.5	12
13	Resorcinol	In	29	In	29
14	*tert*-Butylhydroquinone	11	14	6.0	15
Isothiocyanates					
15	Sulforaphane	0.43	3	0.21	4
16	Benzyl isothiocyanate	0.70	4	3.7	11
17	Phenyl isothiocyanate	In	29	In	29
Peroxides					
18	Hydrogen peroxide	210	27	560	28
19	*tert*-Butyl hydroperoxide	29	23	140	24
20	Cumene hydroperoxide	21	21	210	26
Mercaptans					
21	(±)-2,3-Dimercapto-1-propanol	26	22	12	19
22	3-Mercaptopropane-1,2-diol	In	29	In	29
23	1,2-Ethanedithiol	15	17	21	21
24	2-Mercaptoethanol	180	26	170	25
25	(±)-1,4-Dithiothreitol	In	29	In	29
Trivalent arsenicals					
26	Phenylarsine oxide	0.047	1	0.057	2
27	Sodium arsenite	11	14	2.4	10
Heavy metal salts					
28	$HgCl_2$	1.9	6	0.52	6
29	$CdCl_2$	7.3	10	11	18
30	$ZnCl_2$	73	24	220	27
31	Phenylmercuric chloride	2	7	0.12	3
32	*p*-Chloromercuribenzoate	9.2	13	1.1	8
Other inducers					
33	1,2-Dithiole-3-thione	15	17	1.0	7
34	*β*-Naphthoflavone	0.051	2	0.029	1

Rank order refers to potencies. When two compounds were equipotent they were assigned the same rank, and the subsequent rank was omitted. Inactive (In) is defined as less than a 20% increase in the induction ratio (treated/untreated) at the highest concentration at which there was less than 50% cell toxicity. *(Structures are shown at top of next column.)*



differences in the inducer response of the mouse and rat enhancers can be attributed to this change requires further mutation and deletion experiments.

Comparison of Potencies of Inducers in Enhancing GH Production in Hep G2 Cells Transfected with p41-284GH and in Elevating QR Activities in Hepa 1c1c7 Cells. To determine whether the transcriptional activation mediated through the 41-bp enhancer element accounted for the entire phase 2 enzyme induction produced by all classes of inducers, we compared the concentrations of inducers required to double GH production (CD_{GH}) and QR activity (CD_{QR}) in the two systems. Typical response curves for sulforaphane (0–10 μM) are shown in Fig. 3*A* (QR induction) and Fig. 3*B* (GH production). Notably, the response ratios at high concentrations of sulforaphane (6 μM) were much higher in the GH assay (22-fold) than in the QR assay (6.4-fold). These graphs generated $CD_{QR} = 0.21 \pm 0.05$ μM and $CD_{GH} = 0.42 \pm 0.18$ μM for sulforaphane (Table 2).

An extraordinary diversity of chemical compounds are active in both systems. Various chemical classes of compounds were tested (Table 2): (*i*) *Michael reaction acceptors* (olefins conjugated to electron-withdrawing functions). As shown for QR induction (12, 13), the potency orders for GH production paralleled the electrophilicity of these compounds. For example, 1-nitro-1-cyclohexene ($CD_{GH} = 0.98$ μM; $CD_{QR} = 0.46$ μM), with the olefin conjugated to the powerful electron-withdrawing nitro group, is much more potent in both systems than coumarin (inactive) which is an olefinic lactone. (*ii*) *Diphenols.* Oxidizable diphenols—hydroquinone, catechol, and *tert*-butylhydroquinone—were all comparably potent in both systems, whereas the nonoxidizable resorcinol was inactive (30). (*iii*) *Isothiocyanates.* Sulforaphane was very potent, benzyl isothiocyanate less potent, and phenyl isothiocyanate inactive (12). (*iv*) *Peroxides.* These compounds were all weakly active. Cumene hydroperoxide was slightly more active than *tert*-butyl hydroperoxide, and both compounds were considerably more active than hydrogen peroxide, which induced weakly in both systems (13). (*v*) *Mercaptans.* Mercaptans (which are not electrophiles) were especially active when two thiol groups were adjacent, as in 1,2-ethanedithiol and 2,3-dimercapto-1-propanol (14). 2-Mercaptoethanol was only weakly active, and both dithiothreitol and 3-mercaptopropane-1,2-diol were inactive. Thus two adjacent thiol groups appear to lead to significant inductive potency (14). (*vi*) *Trivalent arsenicals.* Phenylarsine oxide was the most potent inducer tested and was very much more potent than sodium arsenite (14). (*vii*) *Heavy metal salts.* $HgCl_2$, $CdCl_2$, and $ZnCl_2$ were also inducers, with potencies decreasing in this order, which parallels their binding affinity for sulfhydryl groups (14). (*viii*) *Other inducers.* The metabolizable polycyclic aromatic hydrocarbon *β*-naphthoflavone, a bifunctional inducer, was also a very potent transcriptional activator, doubling the GH production at a concentration of only 0.051 μM. Furthermore, 1,2-dithiole-3-thione also enhanced transcriptional activation through the same enhancer element.

Table 2 shows that 6 of 34 compounds from the eight chemically dissimilar classes were inactive in both systems and none was inactive in only one system. The remaining 28 active inducers ranged in potencies over nearly four orders of magnitude from phenylarsine oxide (CD_{GH} = 0.047 μM; CD_{QR} = 0.057 μM) to hydrogen peroxide (CD_{GH} = 210 μM; CD_{QR} = 560 μM), and many compounds were nearly equipotent in the two assay systems. A plot of potencies of QR induction with respect to potencies of GH production for all active inducers (Table 2) gave a good linear correlation, with an r value of 0.89 and a slope of 0.89 (Fig. 4). We conclude that the induction of QR by all of the very different types of inducers is probably mediated entirely through the 41-bp enhancer element and that GH production and QR induction are controlled by the same or very similar rate-limiting processes. Furthermore, comparison of absolute CD values in the two assays gave a linear correlation (r = 0.64), and the slope of the correlation line was 1.17, indicating that the compounds were nearly equipotent in the two assays.

Conclusions. We have demonstrated that a 41-bp enhancer element from the 5′ upstream region of the mouse glutathione transferase Ya gene (20) is responsive to a wide variety of xenobiotic compounds that also induce phase 2 detoxication enzymes in cultured cells and in animals. Transcriptional activation through this element accounts for most, if not all, of the enzyme elevations produced by these inducers. The inducers belong to many different chemical classes; most contain electron-deficient centers and their potencies parallel the strengths of the electron-withdrawing functions. Furthermore, inducers are also substrates for glutathione transferases, thus emphasizing their electrophilicity (13). Paradoxically, dimercaptans were also found to be inducers. The only apparently universal property of all inducers is their capacity for reaction with sulfhydryl groups (by oxidoreduction or alkylation). We suggest, therefore, that a mechanism involving protein thiol modifications modulates the transcriptional activations mediated by the 41-bp enhancer element. In this connection, it is of considerable interest that the redox state of sulfhydryl groups has been implicated in AP-1 binding to DNA (31–33).

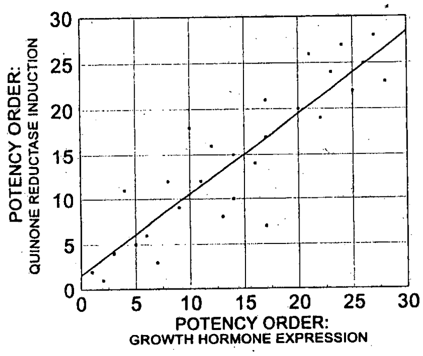


FIG. 4. Order of potencies of 28 compounds in inducing QR (CD_{QR}) and in stimulating growth hormone production (CD_{GH}). The 28 active compounds (Table 2) were ranked from 1 to 28 in order of their potencies in the QR (ordinate) and GH (abscissa) assays. Inactive compounds were excluded. There is a good linear correlation (r = 0.89 and slope = 0.89).

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